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Ethanol Production by Alcohol Tolerant Yeasts Using Different Carbohydrate Sources

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Abstract: This study aimed at comparing the ability of two indigenous yeast species; *Pichia kudriavzevii* strains GY1 and L9 with a strain of *Saccharomyces cerevisiae*, to consume sugars (fructose, galactose, glucose, lactose, sucrose and molasses) and to convert them into ethanol during fermentation. Yeast extract (6g/L), peptone (10g/L), malt extract (6g/L) broth was supplemented with different concentrations (5g/L, 10g/L, 20g/L, 30g/L) of fructose, galactose, glucose, lactose and sucrose respectively. Sugar utilization post incubation for 96 hours at 120 rpm, 30 degree Celsius (°C) was measured using a refractometer. The alcoholic yield using molasses for *Pichia kudriavzevii* strain GY1 10±0.2 (mg/ml) was significantly higher than that of *Pichia kudriavzevii* strain L9 (4±0.2 mg/ml) and *Saccharomyces cerevisiae* strain T (5±0.2 mg/ml) at 96 hours. Strains that produced highest concentration ethanol was *Pichia kudriavzevii* strain L9 in 3.0% (v/v) galactose and fructose respectively, which measured at 7.1±0.48 (mg/ml) and 12.2±0.64 (mg/ml). All studied isolates produced the same amount of ethanol 9.1±0.52 (mg/ml). The use of highly adaptable non Saccharomyces yeast species to a variety of sugars in the pursuit of enhanced ethanol production creates a unique prospective for large scale industrial applications.

Keywords: Alcoholic Fermentation, *Pichia kudriavzevii,* Fructose, Glucose, Galactose, Lactose, Sucrose

1. Introduction

Bioethanol as a renewable energy source is becoming more important in society particularly because of growing concerns with the use of fossil fuels coupled with the impact it has to the environment [1]. Its (bioethanol) production based on the use of renewable feedstock such as agricultural products involves the use of competitive technologies to meet different energy market demands [2]. Targeted renewable sources for ethanol production like sugar cane are those that are rich in fermentable sugar content, thereby making it an ideal substrate for ethanol production. In Brazil, this factor is being exploited for commercial production of ethanol [3], [4]. In Nigeria, the adaptation of this strategy would mean the use of our commercially available resources like molasses to produce sufficient quantity of ethanol to meet growing demands. Utilization of organic waste materials towards the generation of bioethanol could be employed; an approach that underlines the eco-friendly energy theme of the 21st century [5]. Glucose is a good source of carbon, present in a lot of organic material, which is involved in many metabolic processes because a study revealed that the consumption of other sugars only takes place during glucose repression in microbial cells [6]. In the glycolytic pathway, glucose is utilized directly by yeast cells in order of priority in comparison to other sugars like galactose that requires conversion to metabolic intermediates within the

glycolytic pathway prior to use [7]. However, the conversion of glucose to other sugars like fructose within that pathway may have resulted in the adaptation of cells to different sugar mediums. The utilisation of alternate carbon sources maybe through the direct or indirect repression of genes cascaded by genes involved in sugar metabolism [8]. Where we have a range of organic material that could serve as potential fermentation starting material, a time-saving approach would be to access the ability to ferment the key sugar derivatives within such materials. This could be streamlined to but not limited to glucose, fructose, galactose and sucrose [9]. A cost-effective method of producing ethanol involves the use of ethanol-tolerant yeasts. The benefit associated with ethanol production from such adapted species includes an increase in ethanol productivity coupled with the ease of product recovery [10]. Traditionally, strains of *Saccharomyces cerevisiae* serve as the most revered strain of yeast employed for ethanol production in industry although considerable attention has created a search for other yeast species with unique fermentation capabilities [11]. Recombinant DNA technologies has enabled the identification of alternative, non-*Saccharomyces* yeast species that serve as versatile alternatives [12]. In a recent study, we found that non-*Saccharomyces* yeast species with 97-98% sequence homology to *Pichia kudriavzevii*, were capable of normal growth and metabolism in the presence of increasing amounts of ethanol (0-20% v/v) in the broth culture [13]. The paradigm shift towards the application of non-conventional yeast strains is evident with *Kluyveromyces marxianus* which was found to be to grow at extreme temperatures (45-52 degree Celsius °C) in additional to its inherent ability to utilise a wide range of carbon sources [14-16]. Attempts to produce ethanol with *Pichia sp.,* has been attempted with sugarcane juice, glucose and galactose in India [7]. However, not much has been covered in terms of accessing the ethanol production ability of indigenous non-Saccharomyces yeast species in Nigeria using a range of commercially available feedstock like molasses and other simple sugars, the later which represents the major carbon sources in numerous waste or organic materials. The aim of this study was then to analyze the fermentative capability of two indigenous yeast isolates; *Pichia kudriavzevii*, previously obtained from local distillation and milling sites in Nigeria.

2. Materials and Methods

2.1. Ethanol Production Using Molasses

A loop of each isolate obtained from previous experiments was used to inoculate 100ml of autoclaved yeast extract, peptone, dextrose (YPD) broth in 250ml Erlenmeyer flasks. The flasks were incubated at 30 degree Celsius (°C), 120rpm for 24 hours. 10ml of each yeast cell suspension was transferred into 125ml broth composed of 6g/L yeast extract; 10g/L peptone; 6g/L malt extract; 2g/L glucose media. The pH of each medium was adjusted to 5.5. 25mls of autoclaved molasses was then introduced into each flask under the laminar flow hood. The flasks were then incubated for 96 hours at 30°C, 120rpm. At 24 hour intervals, samples were collected to measure sugar utilization and ethanol production. UV spectrophotometry was used to measure yeast growth. All experiments were performed in triplicate and the data reported is the average of the three replications.

2.2. Sugar Uptake

Overnight cultures (10ml in YPD broth) were used to inoculate flasks containing YMP (6g/L yeast extract; 10g/L peptone; 6g/L malt extract) media supplemented with different concentrations (5g/L, 10g/L, 20g/L, 30g/L) of fructose, galactose, glucose, lactose and sucrose respectively. The growth kinetics was characterized via absorbance measurements (OD_{580}) after 4 days to ensure a reasonable degree of fermentation. Sugar utilization was measured using a refractometer.

2.3. Statistical Analysis

All the experiments were conducted in triplicate and analysed using one way ANOVA.

3. Results

Indigenous strains were grown and used in the fermentation of molasses against the control; Fali over a period of 4 days at 28°C. Results from the distillation process at 24 hourly intervals are given in figure 1 representing 3 biological replicates.

The indigenous isolates including the foreign control strain were positive producers of ethanol using molasses for fermentation. Among the indigenous isolates, strain OY (*Pichia kudriavzevii* strains GY1) produced the highest yields of ethanol, ≥ 2X the volume produced by the control sample (*Saccharomyces cerevisiae* strain T) throughout the 4 day batch process. Strain SY (*Pichia kudriazevii* strain L9) produced the least amount of ethanol, < the amount of ethanol recovered from the control strain at any point during the 96 hour interval.

In the sugar utilisation test, the final quantity of the respective concentrations (5g/L, 10g/L, 20g/L, 30g/L) of individual sugars (fructose, galactose, glucose, lactose and sucrose) were measured to ascertain a baseline that would be used to better measure the utilisation of the different sugars by the indigenous and foreign strains for growth and survival over a 96 hour incubatory period figure 1A). At all concentrations of fructose, strain OY (*Pichia kudriavzevii* strains GY1) consumed the highest amount of sugar in comparison to the control strain; Fali (*Saccharomyces cerevisiae* strain T). Stain SY (*Pichia kudriazevii* strain L9) was the least tolerable strain to fructose in comparison to Fali over the studied period. The overall consumption rate of the different concentrations of Galactose is depicted in figure 1B. In comparison to the

baseline value (before fermentation), strain OY and Fali both consumed the highest rate of galactose at 0.5g/L but as the concentration of this sugar increased, different readings were obtained for the different isolates studied. These values coincide with the volume of ethanol obtained for the corresponding sugar (figure 2B). At 30g/L galactose, the maximum ethanol yield was attained by stain SY which in comparison to its sugar utilisation was also the highest amongst the studied isolates.

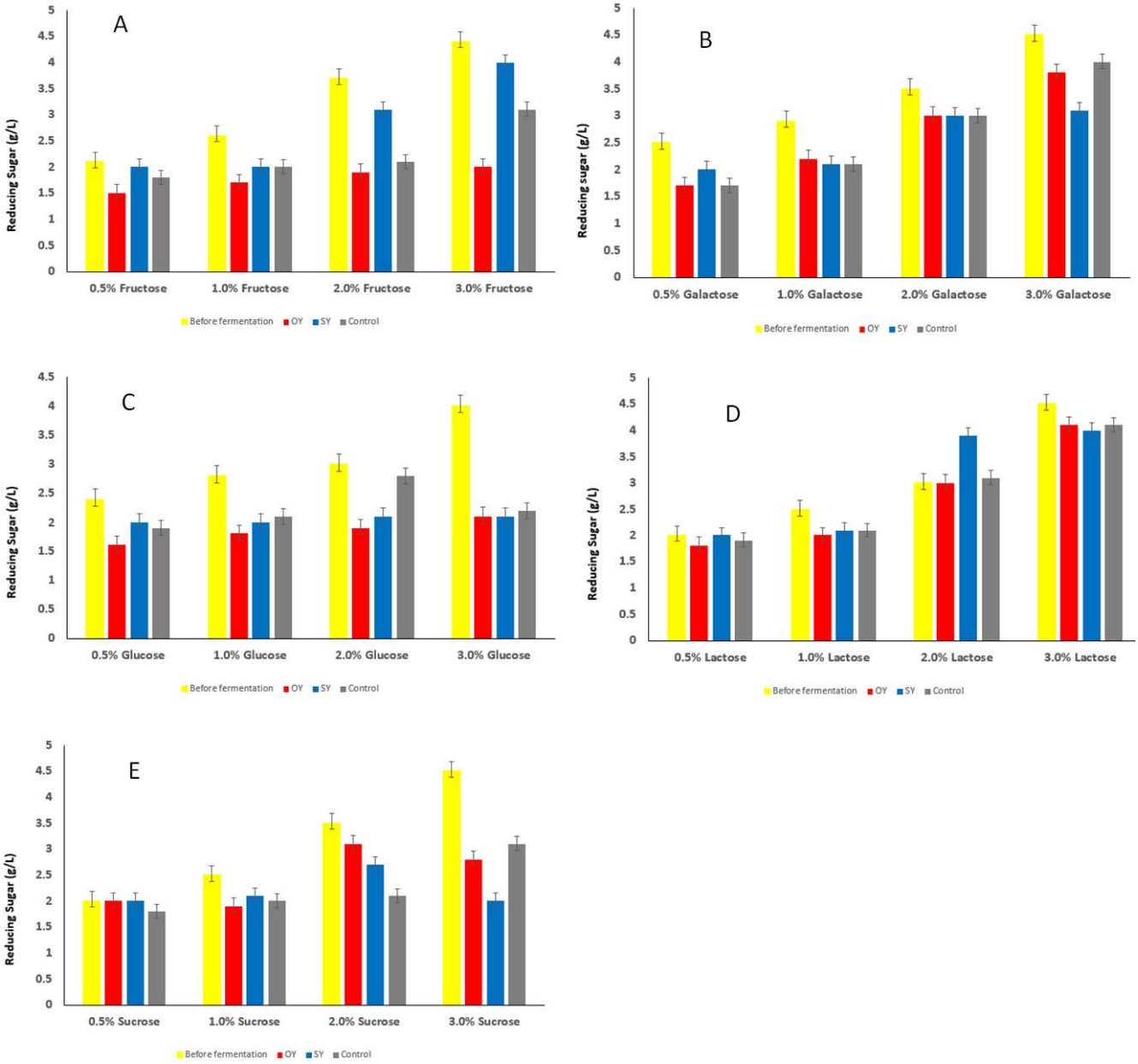

Figure 1. *A-E. Graphical representation of variation in reducing sugars before and after fermentation for YPM medium with variable% of fructose, galactose, glucose, lactose, sucrose.*

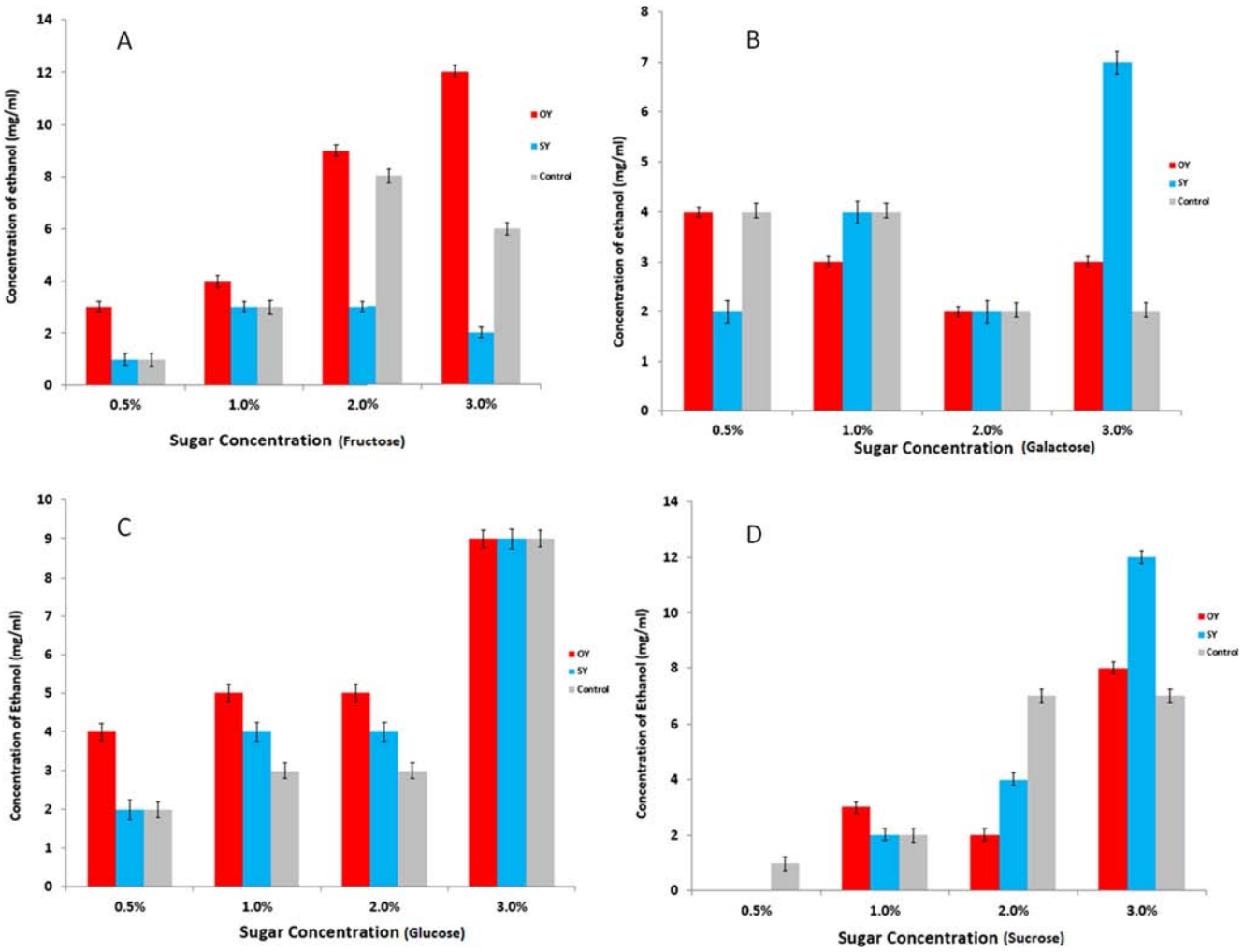

Figure 2. *A-D Graphical representation of ethanol production at different concentrations of sugar after 4 days of fermentation.*

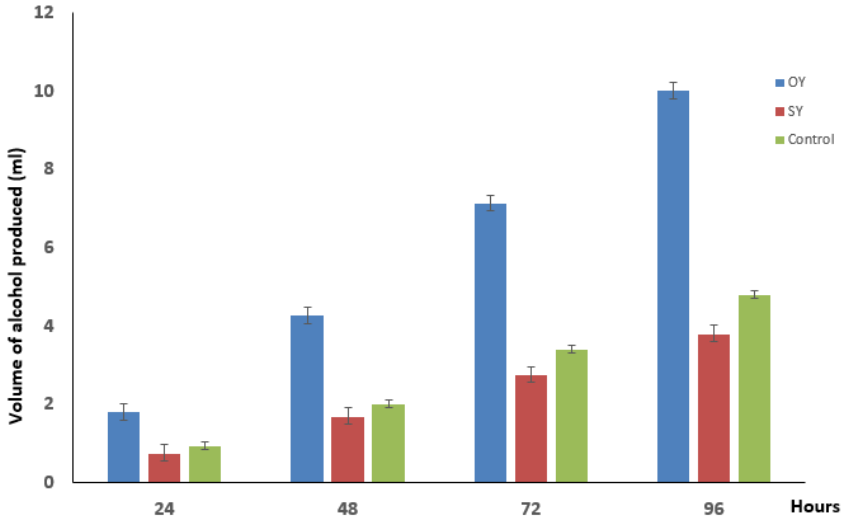

Figure 3. *Ethanol production of yeast isolates. OY = Pichia kudriavzevii strain GY1, SY = Pichia kudriavzevii strain L9, Control = Saccharomyces cerevisiae strain T5.*

4. Discussion

The ability to consume a wide spectrum of sugar medium presents a new set of challenges within industry. The choice of substrate to use as raw materials becomes a welcomed problem to manufacturers particularly in the fermentation industry where the adaptation of yeast cells to such diverse carbon medium results in enhanced production of

metabolites, biomass and ultimately ethanol production. It has been reported that *Saccharomyces cerevisiae*, used in this study as the control (Fali) has the inherent ability to produce high amount of ethanol, whereby it can withstand the onslaught of inhibitory compounds associated with the high yield of the product [9]. However, another reported has shown that incomplete alcoholic fermentation from different substrates such as fructose and glucose is the norm using this yeast specie, [17]. With a strong relationship between sugar consumption and alcohol production, it can be deduced that ethanol production would be better best achieved from high fructose feedstock using strain OY; *Pichia kudriavzevii* strains GY1 (figure 1A, 2A). Fructose consumption this yeast cell increased proportional to the increase in sugar concentration. Compared to the control strain; *Saccharomyces cerevisiae* strain T (Fali), strain GY1 exhibited a higher consumption rate of fructose as the sugar concentration increased, while *Pichia kudriavzevii* strains L9 showed the least rate of consumption (figure 1A, 2A).

Adaptation to galactose is rare and all yeast cells in this study possess low malleability towards the use of this sugar as a sole carbon source (figure 1B, 2B). With increased concentrations of this sugar, *Pichia kudriavzevii* strain L9 revealed the highest level of adaption to galactose than all other strains studied. In-depth analysis would be required to understand this phenomenon before this yeast cell can be applied for commercial purposes. Regulating the temperature of the reaction may assist in its rate of consumption and ethanol production.

From the data obtained, the indigenous yeast cells in this study show a good level of glucose uptake in comparison to the foreign strain (figure 1C). In comparison to the fructose medium, the consumption of sugar by *Pichia kudriavzevii* strains GY1 remains the highest among all studied cells. In general, all yeast cells consumed a higher amount of glucose with increasing concentration. As a negative control measure, varying concentrations of lactose was used in this study. As expected, all yeast cells showed poor adaptation to lactose and its consumption was less than any of the studied sugar (figure 1D). This study also found that sucrose consumption varies among the cells as the rate of consumption differs as the concentrations increased (figure 1E, 2D).

In general, the data obtained verifies reports from other studies that suggest a fractional bioconversion of sugar into ethanol, resulting in the low yield of ethanol in comparison with the quantity obtained using molasses [18-20]. Isolate OY (*P. kudriavzevii* strains GY1) has shown to be the most adaptive to fructose in this study (figure 1A, 2A) whereby it transformed the largest amount of fructose molecules into ethanol, a phenomenon previously thought to be unlikely as observed with the control [20]. The unique results obtained from *Pichia kudriavzevii* strain L9, pertaining to its rate of consumption of the different sugar types tested, coupled with its high yield of ethanol in comparison with the other *Pichia* and *Saccharomyces* strains tested suggests a peculiar genetic and physiological make-up. Furthermore, research into the alcoholic fermentation of different sugar types has revealed that *Kluyveromyces marxianus* is the best ethanol producing yeast from lactose [14], [21], [22].

The maximum yields of ethanol from the indigenous yeast samples were compared to that of Fali (control/foreign strain) on molasses medium with an initial reducing sugar concentration of 18.3 w/v%. The appropriate ethanol yields for *Pichia kudriavzevii* strains GY1 was significantly higher than that of *Saccharomyces cerevisiae* strain T, producing 10±0.2 and 9±0.2 mg/ml of ethanol respectively (figure 3) at 96 hours. *Pichia kudriavzevii* strain L9 produced 4±0.2 mg/ml compared to the control that yielded 5±0.2 mg/ml of ethanol. This pattern was consistent for the entire duration of incubation and suggests that for the purpose of commercial production of ethanol *P. kudriavzevii* strains GY1 would be more effective for use.

5. Conclusion

This study indicated that indigenous non-Saccharomyces yeasts are capable of growing producing ethanol at room temperature. Since the production of alcohol from the indigenous isolates were significantly as high as the control (foreign) strain, the ethanol-tolerance ability should be beneficial in terms of industrial applications. The study also determined that local feedstock which are high in fructose content would be best utilised as an alternate material should a shortage in supply of conventional raw materials occur. *Pichia kudriavzevii* strains GY1 was found to be the most efficient and effective for ethanol production as compared to others. This further suggests that there are various fermentation parameters that if adjusted could improve or affect the production of ethanol. To the best of our knowledge, this probably is the first study reported study on non-Saccharomyces fermentation of fructose, galactose, glucose, lactose and sucrose by *P. kudriavzevii* in comparison with *S. cerevisiae.*

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